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Note

Identification and Characterization of *Acinetobacter* sp. CNU961 Able to Grow with Phenol at High Concentrations

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An *Acinetobacter* sp., strain CNU961, with a higher tolerance to phenol was isolated, and identified through a set of taxonomic studies and a genetic complementation test. Enzymatic and mutagenic studies found that the strain dissimilate phenol by hydroxylation to catechol followed by an *ortho*-ring cleavage pathway to further mineralize it. The phenol hydroxylase, which is an inducible enzyme and requires NADPH for optimum activity, was not inhibited by phenol at concentrations up to 0.5 mM. The different kinetic behaviors of the enzyme activities on NADPH and on phenol reflected that the phenol hydroxylase of strain CNU961 is a multisubunit allosteric enzyme consisting of heterogeneous polypeptides.

Key words: *Acinetobacter* sp.; phenol tolerance; catechol production; *ortho*-pathway; phenol hydroxylase

The pathways involved in the dissimilation of phenol itself have received special attention,[1,2] because they can serve as good models for biodegradation of structurally related phenolic compounds. Catechol, the key catabolic intermediate of phenol dissimilation,[3] is less toxic then its parental compound and can be widely used in manufacturing novel pharmaceuticals, agricultural chemicals, food additives, and even synthetic plastics.[4] However, development of a microbial process for transformation of phenol into this useful intermediate is still limited since phenol is toxic to many microorganisms by leading to significant changes in the structure and functioning of their membranes.[5] Consequently, isolation of a microorganism able to thrive in a high concentration of phenol will offer attractive opportunities for using the strain as a biocatalyst for the effective transformation as well as elimination of phenol. Therefore, the research described herein was begun to isolate and characterize an *Acinetobacter* sp. able to dissimilate phenol optimally at concentrations which are toxic to other microorganisms.

Several strains of bacteria capable of growing on 10 mM phenol were isolated from an industrial sewage effluent in Kwang-Ju City, South Korea, after one month of enrichment. Among them, a strain that had phenotypic characteristics commonly found in *Acinetobacter*, and that had the most rapid growth rate at the expense of phenol, was selected. The selected strain was characterized by standard taxonomic tests[6] with an API 20E kit (bioMerieux SA, Marcy, l'Etoile, France). Although some results of these tests, such as the production of acetoin from sodium pyruvate, were atypical, comparison of its overall characteristics with standard taxonomic criteria led to the isolate being classified preliminarily as an *Acinetobacter*.[7]

The base composition of DNA from the CNU961 was identified from chromatographic analysis as described previously.[8] The GC content of the CNU961 was approximately 42%, which fell within the range of the GC contents found in other *Acinetobacter* spp., about 20% lower than that of *Pseudomonas* spp. DNA.[9] The genetic relatedness of CNU961 with other members of *Acinetobacter* was examined. A tryptophan auxotroph, *A. calcoaceticus* strain ADP112, was transformed with appropriate genomic DNA by the procedure of Juni.[9] It was shown that DNA from CNU961 was able to transform the strain ADP112 to a prototroph (data not shown). This result indicated that the DNA from CNU961 can complement the mutation(s) in ADP112 to permit tryptophan assimilation, and that both strains are very close at the molecular level such as patterns of codon usage. Therefore, the isolate indeed appeared to be a member of *Acinetobacter*, and was designated strain CNU961.

The growth of CNU961 and the use of phenol were measured in cultures supplied with different levels of phenol. The bacterial growth in each medium was monitored by measuring the optical density of each culture at 600 nm. A method using HPLC (Waters 600 Pump, Milford, MA) was developed and used for direct analyses of residual phenol and catechol. Chromatographic analyses were done on a reversed phase C_{18} column with methanol-1% acetic acid solution (40:60, v/v) as the mobile phase, at a flow rate of 1 ml/min. The compounds were monitored at 254 nm wavelength, and the identities of phenol and catechol were confirmed by HPLC comparison of their retention times and absorbance spectra with authentic standards. The CNU961 grew well with phenol at concentrations up to 15 mM, with complete degradation of phenol within 24 h, as shown in Fig. 1. After 2 days of incubation of the medium containing phenol in an uninoculated control flask, less than 10%

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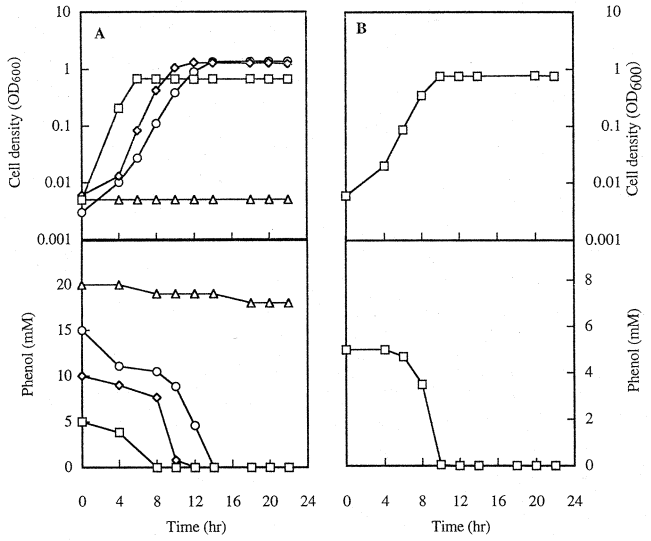

Fig. 1. Growth Kinetics of *Acinetobacter* sp. CNU961 and NCIB8250 Accompanied with the Degradation of Phenol.

Samples removed at indicated times from cultures of strain CNU961 (panel A) and NCIB8250 (panel B) grown with different amounts of phenol were analyzed for growth (top), and residual phenol (bottom). Phenol as a sole carbon and energy source was added to each culture at levels of 5 mM (□), 10 mM (◇), 15 mM (○), and 20 mM (△). For panel B, phenol at concentrations higher than 5 mM did not allow growth of strain NCIB8250.

of the phenol had escaped from the cultures, demonstrating that the major reduction of the phenol in the cultures was caused by biological activity. Recently an *A. calcoaceticus* NCIB8250 able to use phenol as the sole carbon source has been identified and characterized.[1] However, when compared to that of strain CNU961, the growth of strain NCIB8250 was significantly inhibited by phenol above 5 mM (Fig. 1). From this result, in spite of their similarities in catabolism and taxonomy, strain NCIB8250 appeared much less tolerant to phenol especially at high concentrations than strain CNU961.

Apparently, increasing concentrations of phenol in the culture of CNU961 required a prolonged lag phases for adaptation and resulted in a delay in reaching its maximum level of growth. However, the maximum specific growth rate, defined as a specific growth rate at an exponential growth phase, did not decline with the increased level of phenol up to 15 mM (Fig. 1). This indicated that, once adapted, the growth of the CNU961 was not inhibited by phenol up to 15 mM. This phenol tolerance is particularly remarkable when compared to that of many other bacteria. Although Gurujeyalakshmi *et al.*[2] found that one thermophilic *Bacillus* could grow optimally on phenol at the level of 10 mM, phenol dissimilation at this concentration is an unusual characteristic for mesophiles. When 20 mM phenol was added to the culture, however, growth did not occur and significant degradation of phenol was not observed. Apparently, phenol at 20 mM was too toxic to support the growth of CNU961.

Attempts to monitor the transient accumulation of catabolic intermediates from the phenol dissimilation pathway of CNU961 failed. Apparently, the subsequent dissimilation of the intermediates to their final end products occurred so fast and effectively that intermediates did not accumulate. Alternatively, the level of the accumulated intermediates might simply be below the limit of our detection system. In either case, the construction of mutants defective in the phenol dissimilation pathway appeared to be necessary for the accumulation of intermediates. Cells of strain CNU961 washed twice with a 100 mM sodium citrate buffer (pH 5.5), were mixed with 50 μl of N-methyl-N′-nitro-N-nitrosoguanidine solution (MNNG, 1 mg/ml) and incubated to result in 90% death by the procedure of Miller.[10] The cells that survived were washed three times with a 10 mM phosphate buffer (pH 7.6), and were plated on a selective medium, consisting of succinate medium containing 1 mM phenol and 1 mM *p*-toluidine.[11] A mutant strain of CNU961 that could not grow on phenol, but formed dark brown colonies in the presence of succinate, phenol, and *p*-toluidine was isolated and named CNU961M. When both succinate and phenol were supplied, the mutant grew well and accumulated an intermediate identified as catechol (data not shown). This result provides evidence that the first step in the dissimilation of phenol is its hydroxylation to catechol and that phenol hydroxylase is present in the CNU961 strain.

Generally, subsequent oxidation of catechol is catalyzed by a dioxygenase in which both atoms of oxygen are incorporated into the aromatic ring. The catechol is further dissimilated in different strains by either the *ortho-* or *meta*-fission pathway depending on its ring cleavage.[3,12] The catechol dioxygenase activities of CNU961 and CNU961M were examined to further characterize the phenol dissimilation pathway. Enzyme activities were measured using cells from 5-ml cultures grown with different carbon sources, and washed twice using 10 mM phosphate buffer (pH 7.6). The washed cells were resuspended in phosphate buffer, and then disrupted by sonication (Braun Sonic 2000, Germany). The lysates were clarified by centrifugation and then the supernatants were used for enzyme sources. Protein concentrations were measured by the method of Bradford,[13] with bovine serum albumin as the standard. Catechol 1,2- and 2,3-dioxygenase activities were assayed and defined by the way of Kataeva *et al.* and Ngai *et al.*, respectively.[14,15] Both strains had catechol 1,2-dioxygenase activity, but not that of catechol 2,3-dioxygenase (Table 1). The appearance of catechol 1,2-diox-

Table 1. Comparison of Enzyme Activities of *Acinetobacter* sp. CNU961 and CNU961M[a]

Enzyme	Activity (unit/mg protein)[b]	
	CNU961	CNU961M
Phenol hydroxylase[c]	0.750	0.611
Catechol 1,2-dioxygenase[c]	0.422	0.058
Catechol 2,3-dioxygenase[c]	0	0

[a] Cell grown with 5 mM phenol and 10 mM succinate were used as enzyme sources.
[b] One unit is defined as 1 μmole of substrate conversion per minute.
[c] As described in the text.

ygenase activity was taken as evidence that catechol is further oxidized via the *ortho*-fission pathway in CNU961. Like many other strains with the *ortho*-fission dissimilation pathway, the catechol presumably undergoes oxidation to produce *cis,cis*-muconate and the resulting product is further catabolized through β-ketoadipate to tricarboxylic acid cycle intermediates.[12] The observation that the strain CNU961M still can grow with *cis,cis*-muconate as a sole carbon source supports this hypothesis (data not shown).

The activity of phenol hydroxylase was also measured by phenol disappearance in an assay mixture that contained 1 mM NADPH and 0.5 mM phenol in the phosphate buffer. The reaction was started by adding 10 μl of enzyme extract to 1 ml of the assay mixture, and was stopped by a serial addition of stop solutions as described previously.[2] One unit of enzyme activity is defined as 1 μmole of phenol conversion per minute. Phenol hydroxylase activities were observed at similar levels in both CNU961 and CNU961M strains. However, the activity of catechol 1,2-dioxygenase of CNU961M was significantly lower than that of the strain CNU961 (Table 1). Therefore, it was apparent that this partial inactivation of the catechol 1,2-dioxygenase in CNU961M resulted in the accumulation of catechol.

The regulation of phenol hydroxylase was investigated by examining the enzyme activities of CNU961 grown with different carbon sources. Enzyme activity in the organism grown with succinate was not apparent. However, adding phenol to the growth medium induced the activity of the enzyme, a result indicating that the phenol hydroxylase is an inducible enzyme (Table 2). This inducibility of the phenol hydroxylase resembled that reported from other bacteria such as *B. stearothermophilus* and *A. calcoaceticus* NCIB8250.[1,2] However, the induction of enzyme activity was reduced when succinate was also present in the growth medium. This suggested that synthesis of the enzyme would be modulated by one or more additional regulatory mechanisms such as catabolite repression.

The dependence of phenol hydroxylase activity on NADPH or phenol was measured by using the same reaction mixtures with different levels of NADPH or phenol as indicated. NADPH was found to stimulate the activity of the enzyme. Although NADH could replace NADPH, the enzyme activity significantly decreased (data not shown), as in other phenol hydroxylases reported.[16] The enzyme activity revealed hyperbolic dependence on NADPH up to the level of 1 mM, and the

Table 2. Comparison of Enzyme Activities of *Acinetobacter* sp. CNU961 Grown with Different Carbon Sources[a]

Enzyme	Activity (unit/mg protein)[b]		
	Phenol	Succinate	Phenol+Succinate
Phenol hydroxylase[c]	1.016	<0.0001	0.717
Catechol 1,2-dioxygenase[c]	0.421	<0.0001	0.041

[a] Cell grown with 10 mM phenol or 10 mM succinate or both, as indicated, were used as enzyme sources.
[b] Same as Table 1.

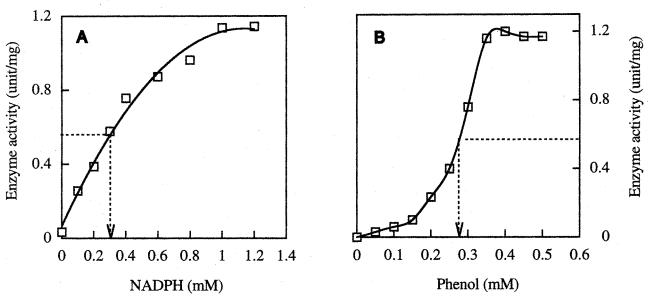

Fig. 2. The Activity Dependency of Phenol Hydroxylase of Strain CNU961 on NADPH and Phenol.
(A) A hyperbolic plot of the enzyme activity as a function of the NADPH concentration. (B) Sigmoidal dependence of the enzyme activity on varied concentrations of phenol. The Km and $K_{0.5}$ values of the enzyme for NADPH and phenol respectively, are indicated by the dotted lines.

Km for NADPH was approximately 0.3 mM (Fig. 2A). The activity dependence on phenol of the enzyme was sigmoidal, and the $K_{0.5}$ for phenol, representing the substrate concentration giving half-maximal enzyme activity, was approximately 0.28 mM (Fig. 2B). Two different types of phenol hydroxylase have been identified: single-subunit enzymes,[2,16,17] and multicomponent enzymes.[1,18] Sigmoidal saturation curves of activity on phenol reflect cooperativity in the phenol hydroxylase of CNU961, which is often observed in allosteric enzymes. Most allosteric enzymes have two or more subunits rather than one single polypeptide. The different patterns of activity dependency of the enzyme on NADPH and phenol strongly suggest that the phenol hydroxylase of CNU961 is a multisubunit protein consisting of heterogeneous polypeptides. Furthermore, there was no substantial inhibition of the enzyme activity by phenol at concentrations up to 0.5 mM (Fig. 2B). This characteristic insensitivity of the enzyme to phenol inhibition differs from that reported in other bacteria[17] and apparently accounts for the growth of the CNU961 in the presence of high concentrations of phenol.

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